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Molecular characterization of fungal endophytic morphospecies isolated from stems and pods of *Theobroma cacao*

J. Crozier[a]*, S. E. Thomas[a], M. C. Aime[b], H. C. Evans[a] and K. A. Holmes[a]

[a]*CABI, UK Centre, Silwood Park, Ascot, Berks SL5 7TA, UK; and* [b]*USDA-ARS, Systematic Botany and Mycology Laboratory, Beltsville, MD 20705, USA*

Endophytic fungi were isolated from healthy stems and pods of cacao (*Theobroma cacao*) trees in natural forest ecosystems and agroecosystems in Latin America and West Africa. These fungi were collected for screening as a potential source of biocontrol agents for the basidiomycetous pathogens of cacao in South and Central America, *Moniliophthora roreri* (frosty pod rot) and *Moniliophthora perniciosa* (witches' broom). Many of these isolates were morphologically unidentifiable as they failed to form fruiting structures in culture, or only produced arthrosporic stages. Affinities with basidiomycetes were suspected for many of these based on colony morphology. Fifty-nine of these morphologically unidentifiable isolates were selected for molecular identification by DNA extraction and sequence analysis of nuclear ribosomal DNA (rDNA). The large subunit (LSU) was chosen for initial sequencing because this region has been used most often for molecular systematics of basidiomycete fungi, and comprehensive LSU datasets were already available for sequence analyses. Results confirmed that the majority of the isolates tested belonged to the Basidiomycota, particularly to corticoid and polyporoid taxa. With LSU data alone, identification of the isolates was resolved at varying taxonomic levels (all to order, most to family, and many to genus). Some of the isolates came from rarely isolated genera, such as *Byssomerulius*, whilst the most commonly isolated basidiomycetous endophyte was a member of the cosmopolitan genus *Coprinellus* (Agaricales). The role of these fungi within the host and their potential as biological control agents are discussed.

Keywords: basidiomycetes, biological control, cocoa, endophytes, rDNA phylogeny, *Theobroma cacao*

Introduction

Chocolate is produced from the fermented and dried beans of the 'chocolate tree' (*Theobroma cacao*, Malvaceae), which has its origins in the tropical rain forests of Amazonia (Motomayor *et al*., 2002; Bartley, 2005). Cacao (cocoa) is now cultivated in most tropical regions throughout the world and is an economically important crop for smallholder farmers (Holmes *et al*., 2004). The main biological constraint to cacao production worldwide is fungal disease (Gotsch, 1997; Bowers *et al*., 2001). The dominant pathogens of cacao in Latin America are *Moniliophthora roreri*, causal agent of frosty pod rot (Evans, 1981) and the closely related *Moniliophthora perniciosa* (= *Crinipellis perniciosa*; Aime & Phillips-Mora, 2005), the causal agent of witches' broom disease (Pound, 1938). In Latin America both diseases are still in an invasive phase (Evans, 2002) and conventional control methods have failed to halt their progress (Evans, 1981; Rubini *et al*., 2005). An effective mechanism of control is urgently required. One alternative strategy being investigated is that of biological control (Holmes *et al*., 2004).

Endophytic fungi exist asymptomatically within host plant tissues for at least part of their life cycle (Wilson, 1995), occupying leaves, stems and branches (Arnold *et al*., 2003; Photita *et al*., 2004; Suryanarayan & Thennarasan, 2004). In woody perennials they are thought to protect the plants in which they live by one or more mechanisms (antibiosis, mycoparasitism, induced resistance and/or competitive exclusion), and are thought to develop from environmental or background inoculum and are not transferred from generation to generation (Johnson & Whitney, 1992). Therefore, plants that have been removed from their natural environment and cultivated are thought to become depleted in their specific or coevolved endophytes (Taylor *et al*., 1999) and, as a result, may become more susceptible to pests and diseases.

*E-mail: j.crozier@cabi.org

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In this study, fungal endophytes of *T. cacao* were isolated and identified as a potential source of novel biocontrol agents for *M. roreri* and *M. perniciosa*. In parallel, studies are being carried out to survey and assess the endophytes within another South American *Theobroma* species, *T. gileri*, the purported original forest host of the frosty pod rot pathogen (Evans *et al*., 2003b). A diverse range of endophytes, primarily anamorphic *Hypocreales* (Ascomycota) and basidiomycetes, were isolated from the woody stems and fruits of *T. gileri* (Evans *et al*., 2003b). These taxonomic assemblages differ from previous studies that found ascomycetes belonging to the Diaporthales, Dothideales, Pezizales and Xylariales to be the predominant endophytic groups (Carroll, 1988; Bills, 1996; Taylor *et al*., 1999; Arnold *et al*., 2001; Vujanovic & Brisson, 2002; Schulz & Boyle, 2005). However, the same profile of fungal endophytes is now being encountered in present studies of cacao as within *T. gileri*, i.e. predominantly anamorphic Hypocreales and basidiomycetes, with the greatest proportion of unidentified morphospecies belonging to the Basidiomycota.

This paper reports on the molecular identification of morphologically unidentifiable endophytic isolates from stems and pods of *T. cacao* in both forest and agroecosystems. Greater emphasis has been placed, at this time, on the classification of the basidiomycetous endophytes, as they occupy a similar ecological niche to that of the basidiomycetous pathogens of cacao (*M. roreri* and *M. perniciosa*) and therefore may be useful as a potential source of biocontrol agents.

Materials and methods

Collection and isolation of endophytic fungi

Surveys for endophytes were carried out between 1999 and 2003, during which time samples were taken from cacao trees (*T. cacao*) in Latin America (Mexico, Costa Rica, Brazil and Ecuador) and West Africa (Cameroon), from both natural forest (Latin America) and cacao farms/germplasm collections (Latin America and West Africa). The method used for sampling the cacao trees and subsequent isolation of endophytes was that described previously by Evans *et al*. (2003a). In brief, a section of living tissue from the inner bark (approx. 8 × 6 cm) was removed from each tree at around chest height (~1·5 m) using a machete and surface-sterilized by flaming with 90% alcohol. The cut surface was then pared further to clean it before 10 triangular slivers were excised from the inner bark using a scalpel blade (Swann-Morton blade no. 11). Each individual sliver was then transferred onto a plate of selective medium: five onto one-fifth strength potato dextrose agar (20% PDA) supplemented with 10 ml L^{-1} penicillin-streptomycin solution (Sigma P0781); and five onto malt extract agar (MEA) supplemented with 0·05 *g* L^{-1} chloramphenicol. Samples were also taken from cacao pods in the field by the method described above after sterilizing the pod surface by flaming with 90% ethanol. On return to the UK, the samples were incubated at 25°C and monitored over an 8-week period. As the fungal isolates began to emerge, hyphal tips or spores were transferred onto 20% PDA or potato carrot agar (PCA) and incubated at 25°C with a near-UV cycle to promote sporulation. Those isolates that failed to form fruiting structures in culture, or only produced arthrosporic stages, were chosen for molecular characterization. All isolates were stored as DIS codes at the CABI UK Centre (Tables 1 and 2). Isolates submitted to GenBank were also deposited in the CABI Genetic Resource Collection (IMI numbers).

DNA extraction, PCR amplification and rDNA sequencing of endophytic morphospecies

Provenances of cultures are detailed in Tables 1 and 2. Hyphal tips from 59 isolates, each representing individual morphospecies groupings based on colony characteristics, were used to inoculate conical flasks containing 60 mL liquid glucose yeast medium (GYM; Mugnai *et al*., 1989), which were incubated at 25°C in an orbital shaker at 100 rpm for 5 days. The resulting mycelium was vacuum-filtered on filter paper (Whatman no. 3) with three washes in sterile distilled water. The mycelium was then freeze-dried before being ground in a pestle and mortar containing liquid nitrogen. The powdered mycelium was stored at −20°C until required. DNA was extracted from the mycelium of the individual isolates and resuspended in 50 μL^{-1} of rehydration solution (1% TE buffer) using a Promega Wizard Genomic DNA Purification Kit.

The first 1 kb of the 28S nuclear ribosomal large subunit (LSU) DNA was chosen for initial amplification and sequencing because this region has been used most frequently in basidiomycete systematics and comprehensively sampled LSU datasets are available for phylogenetic reconstruction and analyses (e.g. Moncalvo *et al*., 2002). Methods for PCR amplification and sequencing followed Aime & Phillips-Mora (2005). Sequences were deposited in GenBank (Tables 1 and 2).

Sequences obtained were initially BLASTed in GenBank (http://www.ncbi.nlm.nih.gov) to predict the family and/or order for each isolate. For closer phylogenetic placement, a data matrix of LSU sequences was then constructed in the following manner: (i) a skeletal LSU dataset was constructed by pruning that of Moncalvo *et al*. (2002) to exclude redundant taxa from lineages not related to any of the fungal endophytes as indicated by BLAST analyses; (ii) additional LSU sequences were then added to this dataset by including all close (> 97% similarity) BLAST results for the isolates; (iii) additional exemplar sequences were included from families and orders of Hymenomycetes to which BLAST analyses indicated the majority of endophytes had taxonomic affinities; and (iv) several heterobasidiomycete sequences were included as outgroups. GenBank accession numbers for additional sequences used in these analyses are shown on Fig. 1. Sequences were manually aligned in Se-Al: SEQUENCE ALIGNMENT EDITOR (Rambaut, 1996). The assembled dataset contained 192 taxa aligned across 928 bp;

Table 1 Identification of Basidiomycota isolates based on large subunit (LSU) rDNA sequence data with geographic location, ecosystem and cacao tissue type from which they were collected, and GenBank accession number

Tentative ID based on phylogenetic analysis of LSU sequence	Isolate code	IMI number	GenBank number	Geographic location	Ecosystem[d]	Tissue
Coprinellus sp. 1	Dis 129a	IMI 393905	DQ327642	Cabiria, CATIE[a], Turrialba, Costa Rica	Exotic	Stem
Coprinellus sp. 2	Dis 238a	IMI 393906	DQ327649	Garzacocha, Rio Napo, Orellana Province, east Ecuador	Forest/exotic	Stem
Coprinellus sp. 2	Dis 112i			Rio Añangu Orellana Province, east Ecuador	Forest	Stem
Coprinellus sp. 2	Dis 222a			Rio Caoni, Puerto Quito, Pichincha Province, west Ecuador	Exotic	Stem
Coprinellus sp. 2	Dis 233d			Rio Añangu, Orellana Province, east Ecuador	Forest	Stem
Coprinellus sp. 2	Dis 251i			Mocache Road, Los Rios Province, west Ecuador	Exotic	Stem
Gloeosterioid sp.	Dis 181c	IMI 393907	DQ327647	CEPLAC[b], Medici Landia, Pará State, Brazil	Forest/exotic	Stem
Corticioid sp. 1	Dis 296a	IMI 393908	DQ327656	Mwellye, Idenao to Mbenge Road, Western Province, Cameroon	Exotic	Stem
Corticioid sp. 1	Dis 296c			Mwellye, Idenao to Mbenge Road, Western Province, Cameroon	Exotic	Stem
Corticioid sp. 1	Dis 296h			Mwellye, Idenao to Mbenge Road, Western Province, Cameroon	Exotic	Stem
Corticioid sp. 1	Dis 298c			Mwellye, Idenao to Mbenge Road, Western Province, Cameroon	Exotic	Stem
Corticioid sp. 2	Dis 168c			Almirante Cacau, Itabuna, Bahia State, Brazil	Exotic	Stem
Corticioid sp. 2	Dis 168d			Almirante Cacau, Itabuna, Bahia State, Brazil	Exotic	Stem
Corticioid sp. 2	Dis 168j	IMI 393909	DQ327645	Almirante Cacau, Itabuna, Bahia State, Brazil	Exotic	Stem
Corticioid sp. 3	Dis 296e	IMI 393910	DQ327657	Mwellye, Idenao to Mbenge Road, Western Province, Cameroon	Exotic	Stem
Phlebioid sp.	Dis 178a	IMI 393911	DQ327646	EMBRAPA[c], Belém, Pará State, Brazil	Forest/exotic	Pod
Podoscypha sp.	Dis 296f	IMI 393912	DQ327658	Mwellye, Idenao to Mbenge Road, Western Province, Cameroon	Exotic	Stem
Corticioid sp. 4	Dis 125b	IMI 393913	DQ327639	Cabiria, CATIE, Turrialba, Costa Rica	Exotic	Stem
Corticioid sp. 5	Dis 298e	IMI 393914	DQ327659	Mwellye, Idenao to Mbenge Road, Western Province, Cameroon	Exotic	Stem
Corticioid sp. 6	Dis 245e	IMI393915	DQ327650	Achidona, Napo Province, Ecuador	Forest/exotic	Stem
Corticioid sp. 7	Dis 292 g	IMI 393916	DQ327655	Mbalmayo, nr. Yaounde, Centre Province, Cameroon	Exotic	Stem
Phanerochaete sp.	Dis 267c	IMI 393917	DQ327652	Rio Caoni, Puerto Quito, Pichincha Province, west Ecuador	Exotic	Stem
Corticioid sp. 8	Dis 267b	IMI 393918	DQ327651	Rio Caoni, Puerto Quito, Pichincha Province, west Ecuador	Exotic	Stem
Oxyporus sp.	Dis 099c	IMI 393919	DQ327635	Chajul, Rio Lacantun, Chiapas, Mexico	Exotic/forest	Stem
Corticioid sp. 9	Dis 267e	IMI 393920	DQ327653	Rio Caoni, Puerto Quito, Pichincha Province, west Ecuador	Exotic	Stem
Byssomerulius sp.	Dis 233h	IMI 393921	DQ327648	Rio Añangu, Orellana Province, east Ecuador	Forest	Stem
Inonotus sp.	Dis 126e	IMI 393922	DQ327641	Cabiria, CATIE, Turrialba, Costa Rica	Exotic	Stem
Hymenochaetoid sp. 1	Dis 140h	IMI 393923	DQ327643	Cabiria, CATIE, Turrialba, Costa Rica	Exotic	Stem
Hymenochaetoid sp. 1	Dis 129b			Cabiria, CATIE, Turrialba, Costa Rica	Exotic	Stem
Hymenochaetoid sp. 1	Dis 131a			Cabiria, CATIE, Turrialba, Costa Rica	Exotic	Stem
Hymenochaetoid sp. 1	Dis 140b			Cabiria, CATIE, Turrialba, Costa Rica	Exotic	Stem
Hymenochaetoid sp. 2	Dis 109d	IMI 393924	DQ327636	El Descanso, Rio Quincha – Rio Napo confluence, Orellana Province, east Ecuador	Forest	Stem
Lentinus sp.	Dis 113e	IMI 393925	DQ327637	Pañacocha – Panayacu Forest, Rio Napo, Orellana Province, east Ecuador	Forest	Stem
Polyporaceae sp. 1	Dis 141d	IMI 393926	DQ327644	Cabiria, CATIE, Turrialba, Costa Rica	Exotic	Stem
Pycnoporus sp. 1	Dis 343d	IMI 393927	DQ327660	Maldonado, Pichincha Province, west Ecuador	Exotic	Pod
Pycnoporus sp. 2	Dis 343f	IMI 393928	DQ327661	Maldonado, Pichincha Province, west Ecuador	Exotic	Pod
Pycnoporus sp. 2	Dis 343c			Maldonado, Pichincha Province, west Ecuador	Exotic	Pod
Polyporaceae sp. 2 (phylotype 1)	Dis 126a	IMI 393929	DQ327640	Cabiria, CATIE, Turrialba, Costa Rica	Exotic	Stem
Polyporaceae sp. 2 (phylotype 1)	Dis 260f			Caluma-Guaranda Road, Bolivar Province, west Ecuador	Exotic/forest	Stem
Polyporaceae sp. 2 (phylotype 2)	Dis 124a	IMI 393930	DQ327638	Cabiria, CATIE, Turrialba, Costa Rica	Exotic	Stem
Polyporaceae sp. 2 (phylotype 2)	Dis 124d			Cabiria, CATIE, Turrialba, Costa Rica	Exotic	Stem
Auriculariales sp.	Dis 290e	IMI 393931	DQ327654	Mbalmayo, nr. Yaounde, Centre Province, Cameroon	Exotic	Stem

[a]Centro Agronómico Tropical de Investigación y Enseñanza.
[b]Commissão Executiva do Plano da Lavoura Cacaueira.
[c]Empresa Brazileira de Pesquisa Agropecuária.
[d]Exotic, cultivated cacao (farm, germplasm collection) outside the centre of origin; exotic/forest, naturalized cacao in a forest habitat outside the centre of origin, e.g. Mayan cacao (brought from the Amazon, centuries, if not millennia ago) is now feral in Mexico and, seemingly, part of the indigenous forest ecosystem (Bartley, 2005); forest, wild cacao within the centre of origin, growing as an understorey tree; forest/exotic, cultivated cacao within the centre of origin, but outside the forest ecosystem.

Table 2 Identification of Ascomycota isolates based on large subunit (LSU) rDNA sequence data with geographic location, ecosystem and cacao tissue type from which they were collected, and GenBank accession number

Tentative ID based on BLAST analysis of LSU sequence	Isolate code	IMI number	GenBank number	Geographic location	Ecosystem[a]	Tissue
Pleosporales sp.	Dis 343g	IMI 393932	DQ327633	Maldonado, Pichincha Province, west Ecuador	Exotic	Pod
Pleosporaceae sp.	Dis 298d	IMI 393933	DQ327632	Mwellye, Idenao to Mbenge Road, Western Province, Cameroon	Exotic	Stem
Hypocreales sp. 1	Dis 256b	IMI 393934	DQ327628	Rio Vinces, Mocache-Vinces Road, Los Rios Province, west Ecuador	Exotic	Stem
Hypocreales sp. 2 (cf. *Leucosphaerina* sp.)	Dis 267a	IMI 393935	DQ327630	Rio Caoni, Puerto Quito, Pichincha Province, west Ecuador	Exotic	Stem
Hypocreaceae sp.	Dis 110g	IMI 393936	DQ327622	Rio Añangu, Orellana Province, east Ecuador	Forest	Stem
Clavicipitaceae sp.	Dis 108h	IMI 393937	DQ327621	El Descanso, Rio Quincha-Rio Napo, Orellana Province, east Ecuador	Forest	Stem
Bionectria sp.	Dis 114e	IMI 393938	DQ327624	Pañacocha – Panayacu Forest, Rio Napo, Orellana Province, east Ecuador	Forest	Stem
Nectriaceae sp. (cf. *Stephanonectria* sp.)	Dis 098a	IMI 393939	DQ327619	Chajul, Rio Lacantun, Chiapas, Mexico	Exotic/forest	Stem
Xylariaceae sp. 1	Dis 190a	IMI 393940	DQ327625	CEPLAC, Medici Landia, Pará State, Brazil	Exotic	Pod
Xylariaceae sp. 2	Dis 343j	IMI 393941	DQ327634	Maldonado, Pichincha Province, west Ecuador	Exotic	Pod
Xylaria sp. 1	Dis 099a	IMI 393942	DQ327620	Chajul, Rio Lacantun, Chiapas, Mexico	Exotic/forest	Stem
Xylaria sp. 2	Dis 112o	IMI 393943	DQ327623	Rio Añangu, Orellana Province, east Ecuador	Forest	Stem
Xylaria sp. 2	Dis 255i			Rio Vinces, Mocache-Vinces Road, Los Rios Province, west Ecuador	Exotic	Stem
Xylaria sp. 3	Dis 233a	IMI 393944	DQ327626	Rio Añangu, Orellana Province, east Ecuador	Forest	Stem
Xylaria sp. 4	Dis 255j	IMI 393945	DQ327627	Rio Vinces, Mocache-Vinces Road, Los Rios Province, west Ecuador	Exotic	Stem
Xylaria sp. 5	Dis 258g	IMI 393946	DQ327629	Rio Vinces, Mocache-Vinces Road, Los Rios Province, west Ecuador	Exotic	Stem
Xylaria sp. 6	Dis 298b	IMI 393947	DQ327631	Mwellye, Idenao to Mbenge Road, Western Province, Cameroon	Exotic	Stem

[a]Exotic, cultivated cacao (farm, germplasm collection) outside the centre of origin; exotic/forest, naturalized cacao in a forest habitat outside the centre of origin, e.g. Mayan cacao (brought from the Amazon, centuries, if not millennia, ago) is now feral in Mexico and, seemingly, part of the indigenous forest ecosystem (Bartley, 2005); forest, wild cacao within the centre of origin, growing as an understorey tree; forest/exotic, cultivated cacao within the centre of origin, but outside the forest ecosystem.

a total of 118 bp were considered too ambiguous to align confidently and were excluded from analyses. Maximum parsimony analyses were conducted in PAUP* 4·0b10 (Swofford, 2002) as heuristic searches with 100 random addition replicates and tree bisection-reconnection (TBR) branch swapping; gaps were coded as missing data. Names for the resulting fungal clades followed Moncalvo *et al.* (2002) and Lutzoni *et al.* (2004).

Results

Sequence data for the first 1 kb of the 5′ end of the 28S nuclear LSU gene for 59 fungal endophyte morphospecies were generated. BLAST analysis revealed that 42 of the isolates were Basidiomycota (Table 1), representing 27 different taxa; the remaining 17 isolates belonged to the Ascomycota and were not analysed further (Table 2).

Basidiomycota were identified by additional parsimony analyses of the basidiomycete sequence data. Of 810 included characters, 103 were variable but parsimony-uninformative and 337 were parsimony-informative. Analyses yielded a single most parsimonious tree [length = 4177, consistency index (CI) = 0·184, retention index (RI) = 0·603] in which all major clades of Basidiomycota were resolved (Fig. 1). Although the topology presented in Fig. 1 was supported by low bootstrapping values, all recovered clades were fully supported in the multigene analyses of Binder & Hibbett (2002) and Lutzoni *et al.* (2004). A single endophyte, DIS 181c, belonged to a clade containing two other taxa currently classified in the Meruliaceae, but which in these analyses appeared associated with the euagarics with weak support. All the endophytic basidiomycetes belonged to the Hymenomycetes; one, DIS 290e, with heterobasidiomycetous affinities, the remainder belonging to various lineages of homobasidiomycetes. Six of these isolates represented two different taxa of euagarics, both belonging to the genus *Coprinellus*, one of which was the most commonly isolated basidiomycetous endophyte in this study; the remainder of the homobasidiomycete isolates belonged to the polyporoid, hymenochaetoid and corticioid lineages. Interestingly, no endophytes were recovered from predominantly ectomycorrhizal russuloid, thelephoroid and boletoid lineages.

Discussion

Of the 854 individual endophyte isolates from the woody tissue of stems or fruits of cacao, 556 isolates (65%) could not be identified on the basis of traditional taxonomic techniques and were grouped into 59 morphospecies. Morphospecies are artificial groupings that are thought not normally to reflect taxonomic relationships (Guo *et al.*, 2003). However, Lacap *et al.* (2003) verified, on the basis of ribosomal DNA sequence analysis, the validity of morphospecies as taxonomic groups. Preliminary identification of the morphospecies in the present study showed that they comprised both Ascomycota and Basidiomycota. The Basidiomycota were the largest group, with 42 of the isolates (representing individual morphospecies), corresponding to 27 different taxa. The basidiomycetes were further characterized as a potential source of biocontrol agents that may occupy similar ecological niches as the basidiomycetous pathogens of cacao *M. roreri* and *M. perniciosa*. The 17 ascomycete morphospecies were not characterized further in this study. Within the Basidiomycota, all but one of the isolates belonged to the homobasidiomycetes, with *Coprinellus* sp. being the most commonly isolated endophyte.

There are limitations to the identification of the basidiomycetes and sterile mycelia using DNA (Guo *et al.*, 2003; Promputtha *et al.*, 2005; Wang *et al.*, 2005). Even if similarities in sequences are high between an isolate and a reference sequence, sufficient data for full resolution are often unavailable. Many of the isolates presented here may be new species or even genera. However, in the absence of fructification it is nearly impossible to confirm the systematic placement of any homobasidiomycete. Additionally, even for a comparatively well-sampled group of fungi such as the homobasidiomycetes, LSU sequence data exist for perhaps less than 10% of known species. Thus, until more reference sequences are available, a confident generic determination for many of these isolates cannot be made. Sequencing of additional genes is under way to aid further identification of the basidiomycete isolates. Identification to genus will be important for determining which isolates may be potential biocontrol agents.

Other endophyte assemblage studies carried out have revealed unidentifiable fungi, as a result of lack of sporulation on artificial culture media (Promputtha *et al.*, 2005; Wang *et al.*, 2005). Many authors have disregarded these isolates and referred to them simply as ‘sterile mycelia’ or ‘unidentified’. Others have grouped such isolates into morphospecies (Fröhlich *et al.*, 2000; Guo *et al.*, 2003; Lacap *et al.*, 2003), as was initially done in this study. Grouping in this way is a useful but limited tool, as comparisons cannot be made with other studies. Very few studies have used molecular techniques to identify these morphospecies further.

Basidiomycetes have only been identified in limited numbers in many of the endophyte studies undertaken, with the majority of endophytes being identified as ascomycetes or their anamorphs (Carroll, 1988; Sridhar & Raviraja, 1995; Bills, 1996; Wang *et al.*, 2005). This could be because they have been overlooked, as most studies have focused on sporulating fungi only, or because of the type of plant tissues sampled. Few previous studies have targeted mature stems as a source of endophytes; other studies have isolated endophytes from leaves (Promputtha *et al.*, 2005) and branches (Chapela & Boddy, 1988; Wang *et al.*, 2005). More specifically, the sampling technique used may also influence the endophytes isolated. It is therefore difficult to estimate whether the number of basidiomycetes isolated from cacao in this study was greater than for other tropical hosts. Although most of the basidiomycetes belong to phylogenetic lineages comprised mainly of wood-rotting fungi, e.g. polyporoid and corticioid,

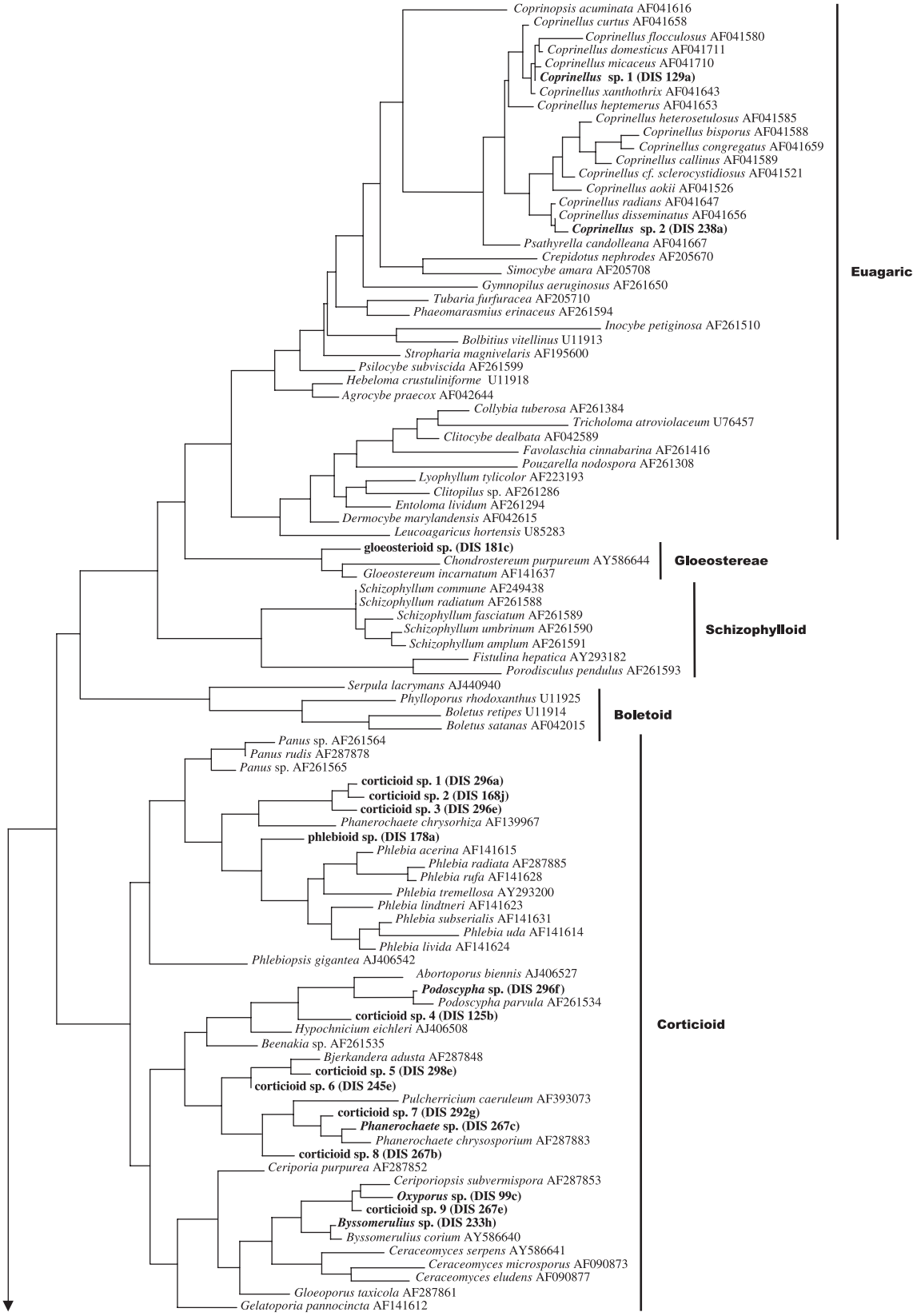

Figure 1 Parsimony analysis of large subunit (LSU) rDNA sequences showing phylogenetic positions of endophytic basidiomycetes within the major lineages of homobasidiomycetes (tree length = 4177, CI = 0·184, RI = 0·603). Clade names for lineages are from Moncalvo *et al.* (2002) and Lutzoni *et al.* (2004). Bold type indicates fungal isolates cultured from inner bark of *Theobroma cacao*.

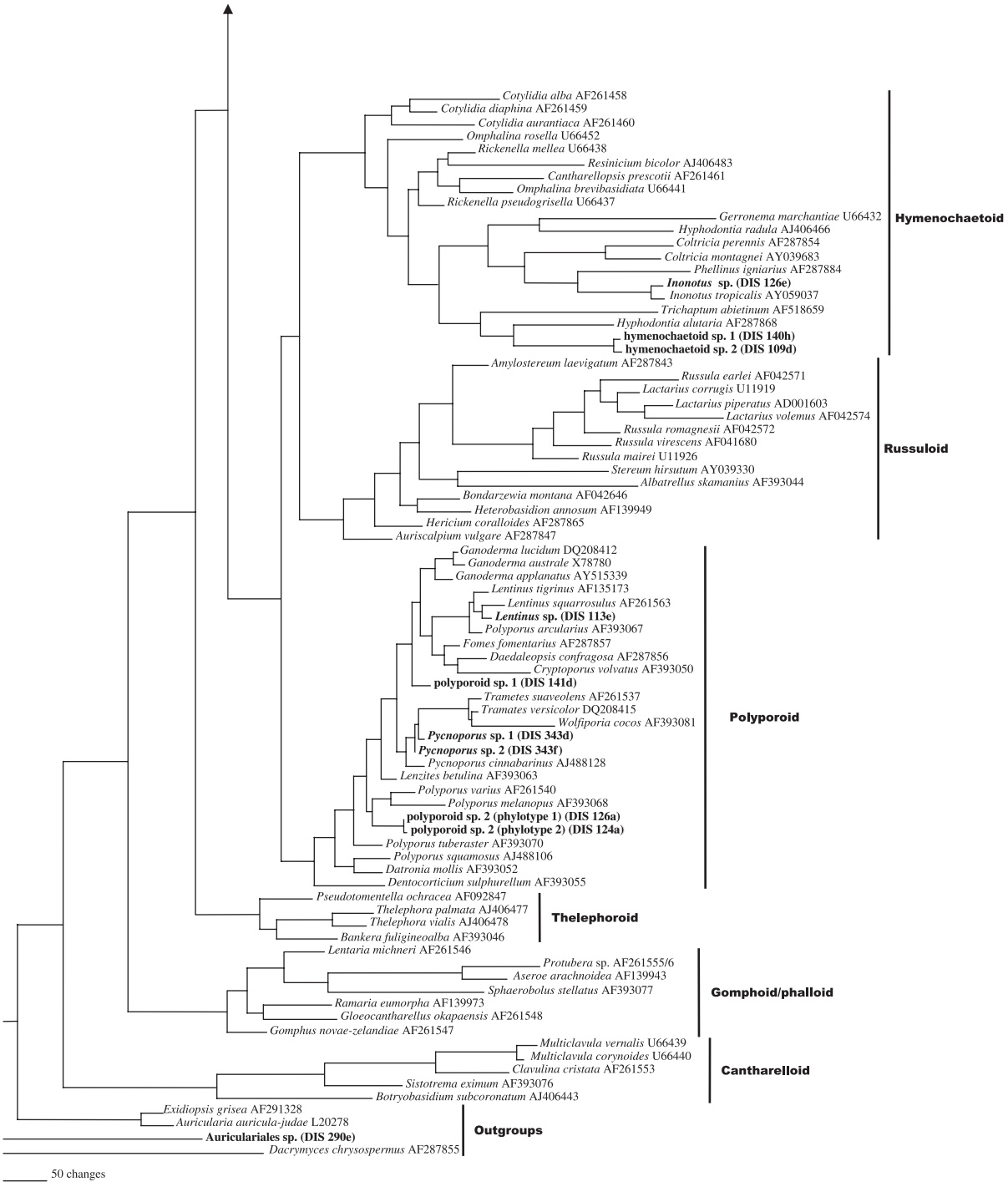

Figure 1 *Continued*

it is thought that these fungi may have adopted an endophytic and asymptomatic phase in their life cycle, in which they exist as 'active' or quiescent colonizers before switching to a saprotrophic phase when the host tissues senesce. This would give them a clear temporal and spatial advantage over less 'sophisticated' wood-degraders.

The rationale for this study, of which this paper forms a part, was to compare and contrast endophytes from within and outside the native range of *T. cacao* in order to determine if there are any unique, potentially coevolved species that could be exploited as classical biocontrol agents for the control of cacao diseases (Evans *et al*.,

2003b). In the case of ascomycetes, promising new species of *Trichoderma* have recently been described from wild cacao (Holmes *et al*., 2004; Samuels *et al*., 2006). However, from the data presented here, there appear to be no such clear and potentially exploitable differences in the basidiomycete assemblages between native and non-native cacao. Only *Byssomerulius* sp., *Lentinus* sp. and the gloeosterioid sp. warrant further investigation. Western and eastern (Amazonian) Ecuador are separated by the Andean Cordillera and it is not surprising therefore that there are marked differences between the basidiomycete colonizers. However, *Coprinellus* sp. 2, which was recorded from several locations in Amazonia, was also present in two distinct localities in western Ecuador (Table 1).

What is evident is that wherever cacao has been introduced, indigenous basidiomycetes have cryptically colonized the stems. In particular, from Costa Rica, where 15 cacao trees were sampled in a germplasm collection, data (unpublished) showed that the majority (65%) of endophytes isolated proved to be basidiomycetes representing a range of clades.

It is not surprising that nine of the isolates were identified as xylariaceous, as these are the most commonly isolated endophytes in tropical regions (Rodrigues & Petrini, 1997). In a similar molecular study by Guo *et al*. (2003), 13 of the 17 morphotypes sequenced were confirmed as members of the Xylariaceae with no basidiomycetes recorded.

Endophytic basidiomycetes could potentially be useful biocontrol agents of the main fungal diseases of cacao in Latin America, as the target organisms themselves are basidiomycetes with a distinct endophytic phase. One key mechanism by which these asymptomatic endophytic basidiomycetes could interfere with the activities of the pathogens is by competing for the same ecological niche. Such an interaction has been observed between *Phlebiopsis gigantea* and *Heterobasidion annosum* (Asiegbu *et al*., 2005). In this way, resident endophytes could prevent initial colonization or displace the invading pathogen. Basidiomycetes are also known to be prolific producers of bioactive metabolites that can be antagonistic towards fungi and other pathogens or pests (Ershova *et al*., 2001; Rosa *et al*., 2003; Zjawiony, 2004). It is possible that by utilizing these mechanisms the basidiomycetes in this study may be active as biocontrol agents. Further studies are required to determine their potential as biocontrol agents of the increasingly invasive and important basidiomycetous pathogens of cacao, *M. roreri* and *M. perniciosa*, in Latin America.

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